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(54) Title: NEUTROPHIL STIMULATING PEPTIDES

VRSSSRTPSD[10]KPVAHVVANP[20]QAEGQLQWLN[30]RRANALLANG[40]

VELRDNQLVV[50]PSEGLYLIYS[60]QVLFKGQGCP[70]STHVLLTHTI[80]

SRIAVSYQTK[90]VNLLSAIKSP[100]CQRETPEGAE[110]AKPWYEPIYL[120]

GGVFQLEKGD[130]RLSAEINRPD[140]YLDFAESGQV[150]YFGIIAL[157]

(57) Abstract

The present invention provides peptides capable of stimulating neutrophils. In particular, the peptides prime neutrophils for a respiratory burst following treatment with N-formyl-L-methionyl-L-leucyl-L-phenylalanine. The peptides have an amino acid sequence substantially corresponding to amino acids 54 to 94 of Figure 1 or a part thereof. These peptides may also be used in the treatment of a subject having depressed neutrophil function.

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NEUTROPHIL STIMULATING PEPTIDES

The present invention relates to peptides having neutrophil stimulating activity, and to use of these peptides as therapeutic agents.

Tumour necrosis factor (TNF) was first identified as a factor found in the serum of Bacillus Calmette-Guerin treated mice which caused haemorraghic regression of certain transplanted tumours and had cytolytic activity on several transformed cell lines in vitro (Carswell et al, PNAS 72, 3666 - 3670; Helson et al, 1975, Nature 258, 731-732). TNF, a product of activated macrophages, has subsequently been shown to be a primary mediator in the pathology of endotoxic shock (Tracey et al 1986, Science 234, 470-474). In addition to its pathological effects TNF also has a central role in host defenses against viral, bacterial and parasitic pathogens.

The cellular targets of TNF important in host defence include neutrophils, eosinophils, monocyte/macrophages and lymphocytes. Within this context TNF is a major mediator of neutrophil activation. TNF stimulates enhanced phagocytosis (Shalaby et al 1985, J.Immunol., 135, 2069-2073), enhanced production of superoxide anions (Teujiimoto et al, 1986, Biochem. Biophys. Res. Commun., 137, 1094-1100), release of lysozyme and hydrogen peroxide and causes neutrophil degranulation (Klebanoff et al, 1986, J.Immunol., 136, 4220-4225). Neutrophils also show enhanced microbicidal and tumouricidal activity when stimulated by TNF (Shalaby et al, 1985, J.Immunol., 135, 2069-2073; Djeu et al, 1986, J.Immunol., 137, 2980-2984; Blanchard et al, 1989, J.Leuk. Biol., 45, 538-545). It has been hypothesized that the cytostatic effect of TNF is mediated by high local concentrations of hydrogen peroxide produced by neutrophils (Shau 1986, J.Immunol., 141, 234-240).

TNF pretreatment enhances the response of neutrophils

to N-formyl-L-methionyl-L-leucyl-L-phenylalanine (F-met-leu-phe) and phorbol myristate acetate through specific receptors (Ferrante et al 1988, Int. Arch. Allergy Appl. Immunol., 86, 82-91). Neutrophils accumulate at sites of inflammation, caused in part by the increased expression of complement receptors by TNF (Berger et al 1988, Blood 71, 151-158). Further TNF causes neutrophil emigration into skin (Cybulsky et al 1988, J. Immunol. 140, 3144-3149).

Neutrophil function is known to be depressed in a number of viral, bacterial and parasitic infections (Abramson and Mills, 1988, Rev. Infect. Dis., 10, 326-341; Ferrante et al, 1989, Immunol. Letts., 22, 301-6). Depressed neutrophil function has, for example, been described in Acquired Immune Deficiency Syndrome (Thorsen et al, 1989, AIDS, 3, 651-653; Ellis et al, 1988, J. Infect. Dis., 158, 1268-1276; Murphy et al, 1988, J. Infect. Dis., 158, 627-630). Clearly TNF, which appears to play an important role in neutrophil activation both in vitro and in vivo as described above, given exogenously has the potential to overcome these neutrophil defects. The administration of TNF or indeed overproduction of TNF is, however, associated with severe side effects and the manifestation of pathology such as thrombocytopaenia, lymphocytopaenia, hepatotoxicity, renal impairment and hypertension.

The present inventors have identified novel peptides derived from the primary amino acid sequence of human TNF which stimulate neutrophil activity. These peptides have indicated that the region of amino acids 54 to 94 of human TNF has previously undiscovered neutrophil stimulating activity. This observation has important clinical applications as treatment with such peptides would be expected to restore depressed or aberrant neutrophil activity, but would not be expected to cause the severe

side effects associated with the therapeutic use of the whole TNF molecule.

Accordingly, in a first aspect the present invention consists in a peptide having an amino acid sequence substantially corresponding to amino acids 54 to 94 of Figure 1 or a part thereof, the peptide being characterized in that the peptide is capable of eliciting superoxide production by neutrophils and of priming neutrophils for an enhanced respiratory burst following treatment with N-formyl-L-methionyl-L-leucyl-L-phenylalanine.

In a preferred embodiment of the present invention the peptide has an amino acid sequence substantially corresponding to amino acids 54 to 94 of Figure 1.

In a preferred embodiment of the present invention the peptide has an amino acid sequence substantially corresponding to amino acids 63 to 83 of Figure 1.

In another preferred embodiment of the present invention the peptide has an amino acid sequence substantially corresponding to amino acids 54 to 68 of Figure 1.

In yet another preferred embodiment of the present invention the peptide has an amino acid sequence substantially corresponding to amino acids 73 to 94 of Figure 1.

In yet a further preferred embodiment of the present invention, the peptide has amino acid sequence substantially corresponding to amino acids 70 to 80 of Figure 1.

As will be appreciated by those skilled in the art from the description which follows the present inventors have demonstrated that the region of human TNF from amino acid 54 to amino acid 94 plays an important functional role in the stimulation of neutrophils. Further, the present inventors have produced 4 peptides namely peptides

304, 308, 309 and 395 (as referred to herein) which have neutrophil stimulating activity.

Armed with this information and with the aid of co-ordinates of the crystalline structure of TNF at 2.6 Å as disclosed by Eck and Sprang, 1989 (J. Biol. Chem., 264: 18795-17605), the person skilled in the art will be able to design non-peptide structures which, in 3 dimensional terms mimic the peptides of the present invention. It is believed that these non-peptide structures will also mimic the physiological effects of the peptides of the present invention. It is intended that such non-peptide structures are included within the scope of the present invention. Changes to the TNF molecule in these regions using e.g. site directed mutaginesis would also be expected to affect neutrophil activation. A schematic representation of the three dimensional structure of TNFα is shown in Figure 4.

Accordingly in a second aspect the present invention consists in a compound the three dimensional structure of which substantially corresponds to the three dimensional structure of the peptide of the first aspect of the present invention, the compound being characterized in that the compound is capable of eliciting superoxide production by neutrophils and of priming neutrophils for an enhanced respiratory burst following treatment with N-formyl-L-methionyl-L-leucyl-L-phenylalanine.

In a preferred embodiment of this aspect of the present invention the compound has a three dimensional structure substantially corresponding to the three dimensional structure of amino acids 63 to 83 of Figure 1.

In another preferred embodiment of this aspect of the present invention the compound has a three dimensional structure substantially corresponding to the three dimensional structure of amino acids 54 to 68 of Figure 1.

In yet another preferred embodiment of this aspect of

the present invention the compound has a three dimensionalstructure substantially corresponding to the three dimensional structure of amino acids 73 to 94 of Figure 1.

In yet a further preferred embodiment of the aspect of the present invention the compound has a three dimensional structure substantially corresponding to the three dimensional structure of amino acids 70 to 80 of Figure 1.

In a further aspect, the present invention consists in a method of treating a subject having depressed neutrophil function, the method comprising administering to the subject a therapeutic amount of the peptide of the first aspect of the present invention.

In a preferred embodiment of the third aspect of the present invention the subject is suffering from acquired immune deficiency syndrome.

Peptide 308, through slective effects on neutrophil degranulation may be administered to individuals suffering from inflammatory syndromes e.g. rheumatoid arthritis, adult respiratory distress syndrome.

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following examples, and Figures in which:-

Figure 1 shows the amino acid sequence of human TNF;

Figure 2 shows the effects of peptides 304 (○), 308 (□) and 309 (△) on the fMLP induced human neutrophil response. Peptides were used at $100\mu g/10^6$ in the 20 min pre-incubation step,

Figure 3 shows the kinetics of the chemiluminescence response elicited by Peptide 395 (●—● ; 395 (50μg) + fMLP; ■—■ ; 395 (50μg); ▲—▲ HBSS + fMLP; ○—○ HBSS); and

Figure 4 is a representation of the TNFα monomer

showing the position of the neutrophil stimulating peptides.

Production of human TNF peptides tested for neutrophils stimulatory activity.

The following peptides were synthesised and are described using the I.U.P.A.C. one-letter code abbreviations for amino acid residues with the TNF sequence region indicated in brackets.

peptide 275

A K P W Y E P I Y L (111-120)

peptide 301

V R S S S R T P S D K P V A H V V A (1-18)

peptide 302

L R D N Q L V V P S E G L Y L I (43-58)

peptide 303

L S A I K S P C Q R E T P E G A (94-109)

peptide 304

L F K G Q G C P S T H V L L T H T I S R I (63-83)

peptide 305

L S A E I N R P D Y L D F A E S G Q V (132-150)

peptide 306

V A H V V A N P Q A E G Q L (13-26)

peptide 307

A E G Q L Q W L N R R A N A L L A N G (22-40)

peptide 308

G L Y L I Y S Q V L F K G Q G (54-68)

peptide 309

H V L L T H T I S R I A V S Y Q T K V N L L (73-94)

peptide 323

T I S R I A V S Y Q T (79-89)

These peptides were synthesised using the following general protocol.

All peptides were synthesised using the Fmoc-polyamide method of solid phase peptide synthesis (Atherton et al, 1978, J. Chem. Soc. Chem. Commun., 13,

537-539). The solid resin used was PepSyn KA which is a polydimethyacrylamide gel on kieselguhr support with 4-hydroxymethylphenoxyacetic acid as the functionalised linker (Atherton et al, 1975, J. Am. Chem. Soc., 97, 6584-6585).

The carboxy terminal amino acid is attached to the solid support by a DCC/DMAP-mediated symmetrical-anhydride esterification.

All Fmoc-groups are removed by piperidine/DMF wash and peptide bonds are formed either via pentafluorophenyl active esters or directly by BOP/NMM/HOBt (Castro's reagent) except for certain amino acids as specified in Table 1.

Side chain protection chosen for the amino acids are removed concomitantly during cleavage with the exception of Acm on cysteine which is left on after synthesis

TABLE 1

Amino acid	Protecting group	Coupling Method
Arg	Mtr or Pmc	Either
Asp	OBut	Either
Cys	Acm (permanent)	Either
Glu	OBut	Either
His	Boc	OPfp only
Lys	Boc	Either
Ser	But	BOP only
Thr	But	BOP only
Tyr	But	Either
Asn	none	OPfp only
Gln	none	OPfp only

Cleavage and Purification

Peptide 302. Peptide is cleaved from the resin with 95% TFA and 5% thioanisole (1.5 h) and purified on reverse phase C4 column. (Buffer A - 0.1% aqueous TFA, Buffer B - 80% ACN 20% A)

Peptide 304. Peptide is cleaved from the resin with

95% TFA and 5% phenol (5 h) and purified on reverse phase C4 column. (Buffer A - 0.1% aqueous TFA, Buffer B - 80% ACN 20% A).

Peptide 308. Peptide is cleaved from the resin with 95% TFA and 5% water (1.5 h) and purified on reverse phase C4 column. (Buffer A - 0.1% aqueous TFA, Buffer B - 80% ACN 20% A).

Peptide 309. Peptide is cleaved from the resin with 95% TFA and 5% thioanisole and purified on reverse phase C4 column. (Buffer A - 0.1% aqueous TFA, Buffer B - 80% ACN 20% A).

In addition, the following synthetic fragments of peptide 309 were synthesized. These peptides had the following amino acid sequence with the TNF sequence region indicated in brackets.

Peptide 393

L T H T I S R I A (76-84).

Peptide 394

S R I A V S Y Q T H V N L L (81-94).

Peptide 395

P S T H V L L T H T I (70-80).

Peptide 396

A V S Y Q T H V N L L (84-94).

Effect of TNF peptides on neutrophil function.

Chemiluminesence assay.

This assay examined the effect of TNF peptides on priming for a neutrophil F-met-leu-phe response as described by Ferrante et al, 1988, (Int. Arch. Allergy Appl. Immunol, 86, 82-91). Purified human neutrophils were pretreated with peptide for 20 minutes before the addition of f-met-leu-phe. The lucigenin dependent chemiluminescence response, which reflects superoxide production, was then measured. The results obtained are set out in Table 2 and are expressed as mV of lucigenin dependent chemiluminescence and represent the maximal cell

activity attained.

In addition, the effects of peptide 304,308 and 309 are shown graphically in Figure 2.

This experiment was repeated with peptides 304, 308 and 309. The results obtained as shown in Table 3.

The experiment was also conducted using peptides 393, 394, 395 and 396. Of these peptides only peptide 395 was able to stimulate the neutrophil respiratory burst (Table 4). The effect of peptide 395 was dose dependent as shown by the results of 3 experiments (Table 5). The kinetics of the chemiluminescence response elicited by peptide 395 is shown in Figure 3. Peptide 395 displays improved solubility over peptide 309.

TABLE 2

Peptide	Concentration $\mu g/10^6$ cells)				
	0	1	10	100	500
275	1.02	0.99	0.69	0.43	0.80
301	0.34	0.93	0.74	0.55	1.10
302	0.37	0.16	0.18	0.29	
303	0.37	0.23	0.17	0.22	
304	0.37	0.18	0.43	2.56	2.76
305	0.37	0.27	0.36	0.24	
306	0.37	0.27	0.35	0.23	
307	0.37	0.35	0.37	0.42	
323	0.37	0.23	0.17	0.47	
308	0.37	0.91	4.80	49.52	
309	0.37	0.38	0.98	13.44	

Results are expressed as mV of lucigenin dependent chemiluminescence and respresent peak of response i.e. the maximal cell activity attained.

TABLE 3

Peptide	Peptide concentration ($\mu g/10^6$ cells)		
	0	10	100
304	0.04	0.36	0.64
304 + fMLP	0.71	0.91	6.97
308	0.04	1.00	11.76
308 + fMLP	0.42	2.74	28.56
309	0.04	0.31	0.69
309 + fMLP	0.42	2.46	12.84

Table 4 Comparisons of 309 and its subpeptides on neutrophil respiratory burst

Treatment (100 ug peptide)	Chemiluminescence (mV)
Diluent	0.58
309	4.70
393	0.31
394	0.33
395	5.32
396	0.70

Table 5 Effect of 395 on neutrophil respiratory burst

Treatment	Chemiluminescence (mV)		
	Exp. 1	Exp. 2	Exp. 3
Diluent	0.58	0.68	0.38
fMLP	1.53	3.53	1.96
1μg 395	3.25	0.89	0.03
1μg 395 + fMLP	3.36	4.55	0.29
10μg 395	4.92	3.97	0.64
10μg 395 +fMLP	7.31	9.10	2.34
50μg 395	8.01	10.81	
50μg 395 + fMLP	12.58	22.09	
100μg 395	2.36	19.14	5.26
100μg 395 + fMLP	5.29	18.10	10.59
100μg 309	5.98	6.68	1.24
100μg 309 + fMLP	27.44	22.77	6.69

Effect on Superoxide Formation

The effect of peptides 308 and 309 on superoxide formation was examined by the cytochrome reduction assay, according to the procedure of Ferrante, 1989 (Infection and Immunity), 57: 2115-2122). The results, expressed as n moles of $O_2/5x10^5$ cells as set out in Table 6.

TABLE 6

Peptide	Peptide concentration ($\mu g/5 \times 10^5$ cells)		
	0	10	100
308	0.270	2.78	4.892
308 + fMLP	2.757	5.00	6.729
309	0.270	0.62	2.30
309 + fMLP	2.757	3.87	5.14

Effect of TNF peptides on neutrophil random migration

Migration of cells is an important property by which cells reach infection sites. Their accumulation at these sites is also dependent on the capacity of inflammatory mediators to inhibit their migration out of the sites. The present inventors have examined TNF and peptide 304, 308 and 309 for their effect on the migration of neutrophils.

In these experiments neutrophils were pre-treated with the peptide or TNF and then examined their ability to migrate out of wells in agarose as described by Ferrante et al, 1988, (Arch. Allergy Appl. Immunol. 86:82-91). The results are shown in Table 7. The results show that TNF was only partially migration inhibitory at 100 units/10^6 cells. Both peptides 308 and 309 were potent migration inhibitors, however, peptide 304 was found to be chemokinetic (it stimulated cell migration).

TABLE 7

Treatment	Inhibition of Migration ($\mu g/10^6$ cells)		
	0	10	100
TNF	ND	ND	4%
304*	-16%	-43%	-883%
308	0	0	100%
309	0	0	100%

*Peptide 304 was found to stimulate (chemokinetic)

Chemotactic properties of TNF and peptides

The chemotactic properties of TNF and peptides 304, 308 and 309 were examined using the following method:

3ml of molten 2% agarose was mixed with 3ml of 2x concentrated medium 199 containing foetal calf serum (10%) and poured into Petri dishes. Sets of 3 wells of 2.5mm diameter, each 3mm apart, were cut in the agarose. 5μl of neutrophils (2×10^5 cells) were added to the inner well, with chemotactic agent or control medium added to the outer wells. Migration at various time intervals was then measured. The results of these experiments are shown in Table 8.

TABLE 8

Agent*	Migration distance (mm) at			
	1.5h		2.5h	
	None	Agent	None	Agent
fMLP	0.50	1.46	0.66	2.45
TNF	0.50	0.48	0.66	0.69
304	0.48	0.47	0.68	0.72
308	0.50	0.66	0.63	1.41
309	0.50	0.53	0.63	0.68

* To the chemotactic well was added 5ml of $1x10^{-7}$ MfMLP, of either peptide 304; peptide 308 and peptide 309 or 10^3 U/ml of TNFα

Effect of TNF Peptides on Neutrophil Degranulation

The conditions of measuring degranulation were as described by Ferrante A, 1989, (Infect and Immunity 57, 3110-3115). In these studies 100μl of neutrophils (10^7/ml) were incubated for 20min at 37 ^{o}C after which 10μl of cytochalasin B was added. After 10 min incubation the volume of cell suspension was made up to 1 ml with Hanks Balanced Salt Solution (HBSS). The cell-free supernatants were collected and analysed for enzyme levels after a further incubation at 37^{o}C. β-Glucuronidase activity was measured fluorimetrically by using 4-methylumbelliferyl-β-D-glucuronide as substrate. This involved incubating 50μl of 2.5 mM substrate in 0.1 M citric acid -sodium phosphate buffer, pH 4.5, at 37^{o}C for 3 h. The reaction was stopped by adding 1.5 ml of 0.2 M glycine-sodium hydroxide buffer, pH 10.7 and the fluorescence of the liberated 4-methylumbelliferone was quantitated by using excitation and emission wavelengths of 336 and 446 nm, respectively. Vitamin B_{12} binding protein was measured using ^{57}Co-vitamin B_{12}. This assay is based on the principle that the binding protein binds to the ^{57}Co-Vitamin B_{12} and as a result the radioactive vitamin B_{12} does not bind to charcoal. The resultant radioactivity in the supernatant can then be equated to the concentration of vitamin B_{12}-binding protein in the sample. The results of these experiments are set out in Table 9 A and B.

Table 9 Effect of TNF Peptides on Neutrophil Degranulation

Neutrophils were treated with 100μg/10^6 cells of 304, 305 or 308±fMLP (in the presence of CytoB).

A.

Treatment	β-glucuronidase release (%) Exp. 1	Exp. 2	Exp. 3	Exp.
HBSS	3.63	1.84	2.72	6.23
HBSS + fMLP	23.62	41.41	40.19	27.54
304	3.63	3.14	2.52	9.82
304 + fMLP	26.95	36.43	35.34	36.65
Control peptide	-	-	-	13.41
Control peptide + fMLP	-	-	-	35.69
308	0.8	0.65	2.76	0.72
308 + fMLP	17.57	28.86	17.86	18.20

B.

Treatment	Vitamin B_{12} Binding Protein Exp. 1	Exp. 2	Exp. 3	Exp. 4
HBSS	9.21	9.27	9.67	4.80
HBSS + fMLP	28.85	27.91	45.31	27.33
304	11.40	10.82	13.06	8.42
304 + fMLP	43.76	35.60	59.15	37.12
Control peptide	-	-	-	7.49
Control peptide + fMLP	-	-	-	38.62
308	2.00	2.08	5.70	2.25
308 + fMLP	35.81	27.59	26.55	21.51

The effects of TNFα peptides on stimulation of neutrophil respiratory burst, degranulation, migration inhibition, chemokinesis and chemotaxis were investigated. As can be seen from the results set out above only peptides 304, 308 and 309 were found to prime human neutrophils for the respiratory burst associated with f-met-leu-phe treatment, i.e. in a manner analogous to that of TNFα . Together these peptides comprise the primary amino acids sequence region of amino acids 54 to 94 of human TNFα . Peptide 308 is a particularly potent primer of neutrophils in this assay.

It is to be noted, however, that peptide 323 which has a sequence which corresponds to amino acids 79 to 89 of human TNF was not found to be capable of priming neutrophils for the respiratory burst associated with f-met-leu-phe treatment. The reason for the lack of neutrophil stimulating activity of this peptide has not as yet been ellucidated, however, one hypothesis for the lack of activity of this peptide may be that peptide 323 does not include the amino acid residues which bind to the TNF receptor on the neutrophils.

Peptides 308 and 309 have also been found to be potent inhibitors of neutrophil migration whilst peptide 304 has been found to be chemokinetic. Peptide 308 has also been found to be strongly chemotactic.

The effects of TNF peptides 304 and 308 on degranulation of neutrophils (Table 9) showed that peptide 308 decreased the release of the contents of both the specific and the azurophilic granules as measured by the release of Vitamin B_{12} binding protein and β-glucuronidase release respectively. This effect of peptide 308 was still apparent following stimulation with fMLP. In contrast, peptide 304 had no effect on neutrophil degranulation in the absence of fMLP. In the presence of fMLP peptide 304 enhanced release from

specific granules but not azurophilic granules.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

CLAIMS:-

1. A peptide having amino acids sequence substantially corresponding to amino acids 54 to 94 of Figure 1, or a part thereof, wherein the peptide is capable of priming neutrophils for a superoxide production and an enhanced respiratory burst following treatment with N-formyl-L-methionyl-L-leucyl-L-phenylalanine.

2. A peptide as claimed in claim 1 in which the peptide has an amino acid sequence substantially corresponding to amino acids 52 to 94 of Fib. 1.

3. A peptide as claimed in claim 1 in which the peptide has an amino acid sequence substantially corresponding to amino acids 54 to 68 of Figure 1.

4. A peptide as claimed in claim 1 in which the peptide has an amino acid sequence substantially corresponding to amino acids 73 to 94 of Figure 1.

5. A peptide as claimed in claim 1 in which the peptide has an amino acid sequence substantially corresponding to amino acids 70 to 80 of Figure 1.

6. A compound having a three dimensional structure which substantially corresponds to the three dimensional structure of the peptide as claimed in any one of claims 1 to 5, in which the compound is capable of priming neutrophils for a respiratory burst following treatment with N-formyl-L-methionyl-L-leucyl-L-phenylalanine.

7. A method of treating a subject having depressed neutrophil function the method comprising administering to the subject an effective therapeutic amount of the peptide as claimed in any one of claims 1 to 5.

8. Method as claimed in claim 7 in which the subject is suffering from acquired immune deficiency sydrome.

9. A method as claimed in claim 7 in which the subject is suffering from cancer.

10. A method of treating a subject suffering from an inflammatory syndrome comprising administering to the

subject an effective therapeutic amount of peptide as claimed in claim 3.

11. A method as claimed in claim 10 in which the inflammatory syndrome is rheumatoid arthritis or adult respiratory distress syndrome.

FIG. 1

VRSSSRTPSD[10]KPVAHVVANP[20]QAEGQLQWLN[30]RRANALLANG[40]

VELRDNQLVV[50]PSEGLYLIYS[60]QVLFKGQGCP[70]STHVLLTHTI[80]

SRIAVSYQTK[90]VNLLSAIKSP[100]CQRETPEGAE[110]AKPWYEPIYL[120]

GGVFQLEKGD[130]RLSAEINRPD[140]YLDFAESGQV[150]YFGIIAL[157]

FIG. 2

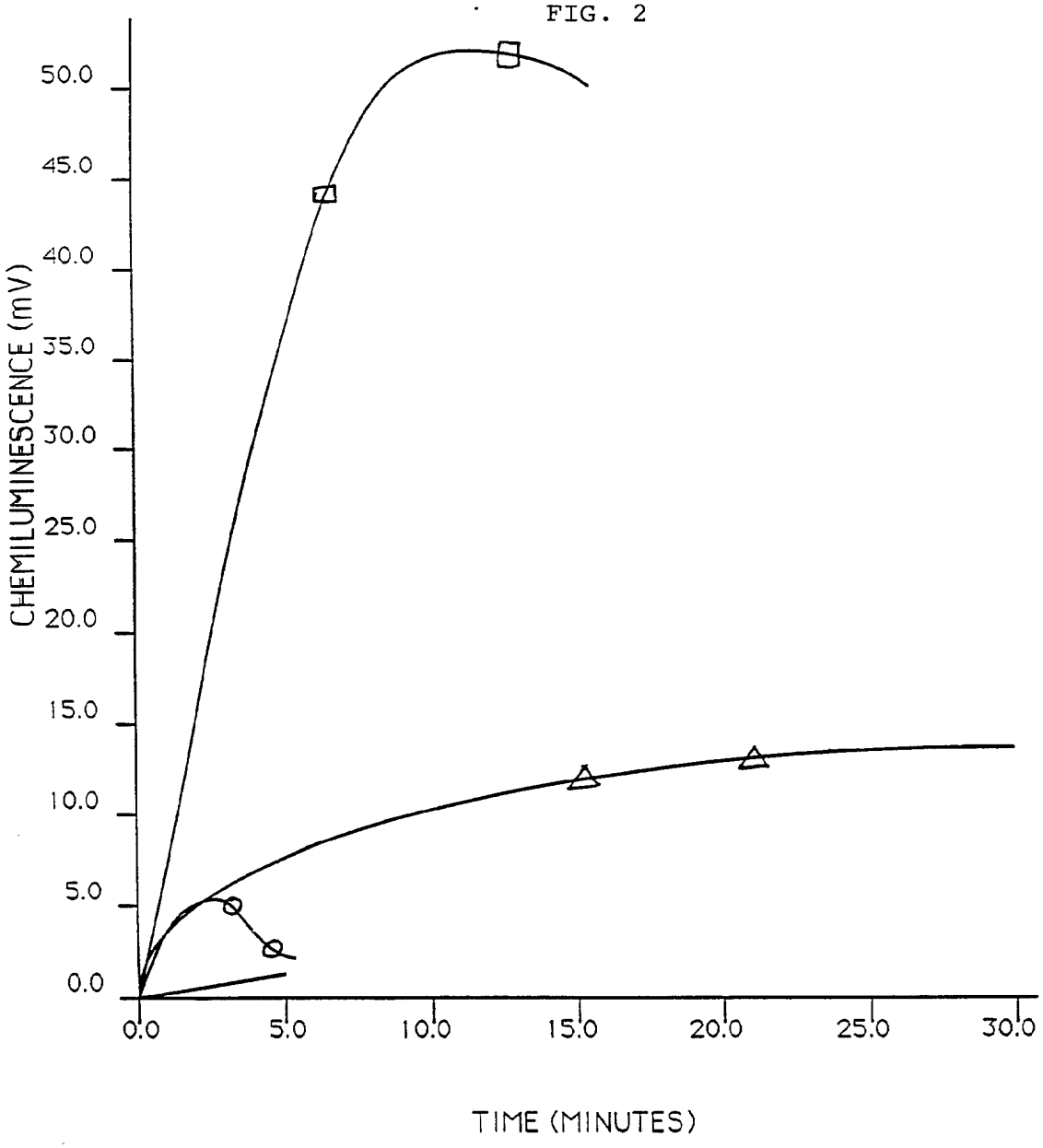

CHEMILUMINESCENCE (mV)
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
TIME (MINUTES)

FIG. 3

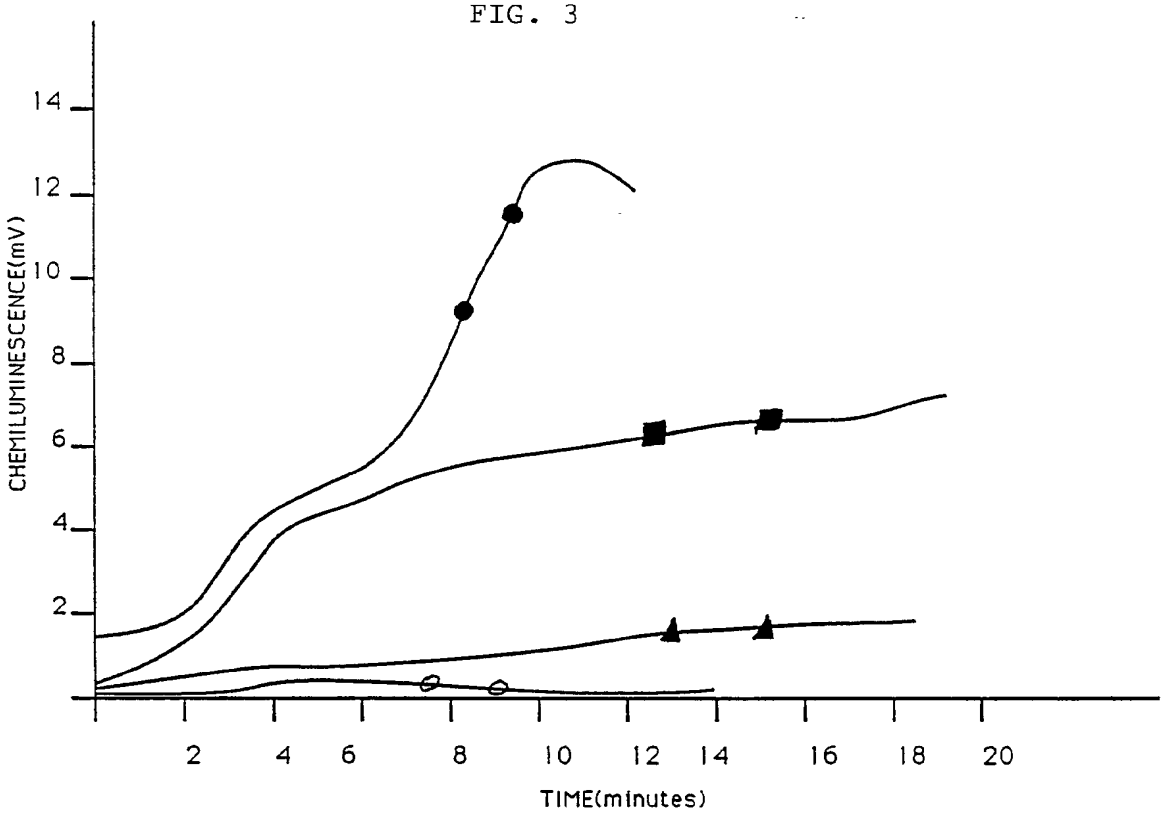
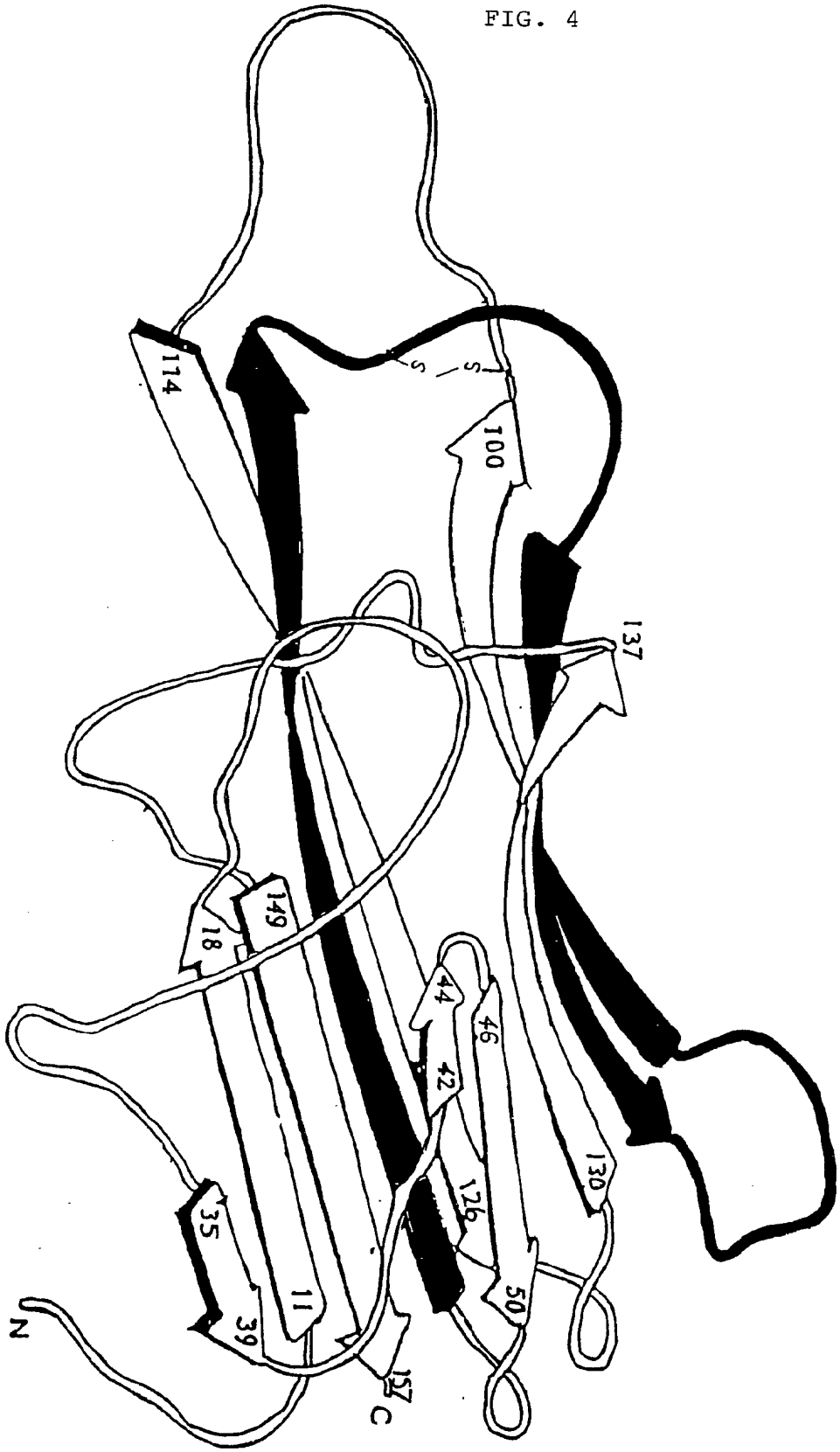

CHEMILUMINESCENCE(mV)
14
12
10
8
6
4
2
2
4
6
8
10
12
14
16
18
20
TIME(minutes)

FIG. 4



INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 91/00086

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[5] C07K 7/06, 7/08, 7/10, A61K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	C07K 7/06, 7/08, 7/10, C07C 103/52
Chem Abs online:	KEYWORDS: Tumor Necrosis Factor OR Tumour Necrosis Factor OR TNF

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

AU : IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
A	AU,B, 44652/85 (GENENTECH, INC.) 9 January 1986 (09.01.86)	
X	Proceedings of the National Academy of Sciences USA, Volume 84, no. 21, issued 1987, November (Washington D.C. USA), S.H. Socher et al. "Antibodies against amino acids 1-15 of tumor necrosis factor block its binding to cell-surface receptor", see pages 8829-8833.	(1,2)

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30 April 1991 (30.04.91)	29 MAY 1991
International Searching Authority	Signature of Authorized Officer
Australian Patent Office	T. SUMMERS

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [ ] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.[ ] Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2.[ ] Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.[ ] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

1.[ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.[ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.[ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 91/00086

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members		
AU 44652/85	BR 8906075	CA 2001560	DK 3058/85
	EP 168214	EP 372740	ES 544843
	ES 557105	FI 852626	HU 38125
	IL 75717	JP 2194878	JP 61040221
	NO 852673	PL 254399	PT 80758
	US 4650674	ZA 8505059	

END OF ANNEX